

p53-Derived Host Restriction of HIV-1 Replication by Protein Kinase R-Mediated Tat Phosphorylation and Inactivation

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ABSTRACT

Tumor suppressor p53 has been suggested to be a host restriction factor against HIV-1 replication, but the detailed molecular mechanism has remained elusive for decades. Here, we demonstrate that p53-mediated HIV-1 suppression is attributed to double-stranded RNA (dsRNA)-dependent protein kinase (PKR)-mediated HIV-1 *trans*-activator (Tat) phosphorylation and inactivation. p53 silencing significantly enhanced HIV-1 replication in infected cells. Ectopic expression of p53 suppressed Tat activity, which was rescued by PKR silencing. In addition, ectopic expression of PKR abolished Tat activity in p53^{-/-} and eIF2α^{CA} cells. Finally, we found that HIV-1 infection activates p53, followed by the induction and activation of PKR. PKR directly interacted with HIV-1 Tat and phosphorylates the first exon of Tat exclusively at five Ser/Thr residues (T23, T40, S46, S62, and S68), which inhibits Tat-mediated provirus transcription in three critical steps: (i) phosphorylation near the arginine-rich motif (ARM) inhibits Tat translocation into the nucleus, (ii) accumulation of Tat phosphorylation abolishes Tat–Tat-responsive region (TAR) binding, and (iii) Tat phosphorylation at T23 and/or T40 obliterates the Tat-cyclin T1 interaction. These five Ser/Thr sites on Tat were highly conserved in HIV-1 strains prevalent in Europe and the United States. Taken together, our findings indicate that p53-derived host restriction of HIV-1 replication is likely attributable, at least in part, to a noncanonical p53/PKR/Tat phosphorylation and inactivation pathway in HIV-1 infection and AIDS pathogenesis.

IMPORTANCE

HIV-1-mediated disease progression to AIDS lasts for years to decades after primary infection. Host restriction and associated viral latency have been studied for several decades. p53 has been suggested as an important host restriction factor against HIV-1 replication. However, the detailed molecular mechanism is still unclear. In the present study, we found that the p53-mediated HIV-1 restriction is attributed to a p53/PKR/Tat-inactivation pathway. HIV-1 infection activated p53, which subsequently induced PKR expression and activation. PKR directly phosphorylated Tat exclusively at five specific Ser/Thr residues, which was accompanied by significant suppression of HIV-1 replication. Accumulation of Tat phosphorylation at these sites inhibited Tat function by blocking Tat nuclear localization, Tat binding to TAR, and Tat-cyclin T1 interaction. Our findings provide a better understanding of the p53-derived host restriction mechanism against HIV-1 replication in AIDS pathogenesis and may contribute to further research focusing on the investigation of potential therapeutic targets for HIV-1.

Human immunodeficiency virus type I (HIV-1) encodes a highly conserved *trans*-activator (Tat) protein, which is essential for viral replication and disease progression (1). Although Tat is a potent *trans*-activator in HIV-1 replication, HIV-1-mediated disease progression lasts for decades after primary infection (2). Host restriction mechanisms have been discussed in association with latent reservoirs, inappropriate provirus integration, epigenetic silencing, absence of host transcription factors for HIV-1 expression, cellular repressors, and insufficient Tat activity (3–5). However, the host restriction of HIV-1 replication and associated viral latency still remains unclear and is a major barrier in the eradication of AIDS. The tumor suppressor p53 has been suggested as an important factor in host restriction of HIV-1 replication (6–8). HIV-1 infection induces upregulation of p53 in primary CD4⁺ T cells (9, 10). It was also reported that HIV-1 infection activates p53 target genes, leading to cell apoptosis (11). However, p53-mediated host restriction of HIV-1 replication is not completely understood.

Protein kinase R (PKR) is a well-characterized double-stranded RNA-dependent protein kinase with important roles in type 1 interferon (IFN)-mediated antiviral activity (12). Recently,

we reported that PKR is a novel p53 target gene which plays a crucial role in the tumor suppressor function of p53 under genotoxic conditions (13) and induces G₂ arrest by triggering Cdc2 degradation (14).

The replicating capacity of HIV-1 strains has been suggested to be closely associated with Tat activity and sequence variations. Tat

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proteins from African strains Mal and Eli are more active than those from Europe and the United States (15). In addition, the natural amino acid substitution T23N in Tat is predominant in clade A, C, D, and G of HIV-1 strains in Africa that are known to be fast-replicating (16). On the other hand, insufficient Tat activity is accompanied by the establishment of HIV-1 latency (17, 18), and latent infection can be reactivated by exogenous Tat (19). Tat activity is altered by cellular modification factors such as p300, p300/CBP-associating factor (PCAF), Hdm2, Hexim1, and SKIP (20–23). Tat phosphorylation by PKR (24) and CDK2 (25) has also been reported, and phosphorylation of Tat by PKR at three Ser/Thr sites (S62, T64, and S68) was shown to increase Tat–Tat-responsive region (TAR) binding and enhance transcription (26). However, the detailed molecular mechanisms underlying host restriction of HIV-1 replication via p53/PKR-mediated Tat phosphorylation and inactivation after HIV-1 infection are largely unknown.

Here, we found that HIV-1 infection activates p53, which subsequently induces PKR expression, followed by significant inactivation of Tat by PKR-mediated Tat phosphorylation at five specific Ser/Thr residues that are highly conserved in European and American HIV-1 strains, independent of the canonical PKR/eIF2 α translational inhibition pathway. PKR-mediated Tat phosphorylation inhibited Tat nuclear localization, Tat binding to TAR, and Tat-cyclin T1 interaction, accompanied by suppression of HIV-1 replication. Our findings indicate that p53 plays an important role in the inhibition of HIV-1 replication via Tat inactivation in patients infected with HIV-1 strains which are predominant in Europe and the United States. This study provides a better understanding of the p53-derived host restriction mechanism against HIV-1 replication in AIDS pathogenesis and may contribute to further research focusing on the investigation of potential therapeutic targets for HIV-1.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies. HeLa, HEK293, C8166, and Jurkat cells were obtained from American Type Culture Collection (ATCC) or the Korean Type Culture Collection (KTCC). Isogenic HCT116 p53^{+/+} and p53^{-/-} cells were kindly provided by B. Vogelstein (Johns Hopkins Oncology Center, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) (GIBCO/BRL). Jurkat-Tat (Tat-expressing Jurkat) cells were kindly provided by J. Sodroski (Dana-Farber Cancer Institute, Harvard Medical School, USA). HeLa/ Δ PKR and Jurkat/ Δ PKR cells, stably transfected with the pSLX-antisense PKR(620–180) plasmid, were selected in the presence of 800 μ g/ml G418 and maintained in medium containing 400 μ g/ml G418. PKR knockdown (PKR^{KD}) HCT116 (p53^{+/+} or p53^{-/-}), HeLa, and Jurkat cells, as well as constitutively active eIF2 α mutant (eIF2 α ^{CA}) cells were prepared by transfection with PKR-targeting short hairpin RNA (shRNA) (sh-PKR) or pSLX-eIF2 α 51A plasmids as reported previously (13). The HIV-1 laboratory strain HXB2, its cDNA clone (pHXB2), and the HIV-1_{IIIB} strain were obtained from the AIDS Research and Reference Reagent Program (ARRRP, NIH, USA) and cultivated as described previously (27). HXB2 cDNAs containing mutant Tat were generated by subcloning the cDNA fragment at NheI/NcoI sites with mutant *tat*-containing fragments after PCR-based mutagenesis on *tat*. pcDNA-p53, pcDNA3-(Flag/Myc)-PKR, pcDNA3-Flag-tat, pGEX-5X-1-PKR, and pEGFPc2-cyclin T1 plasmids were constructed as described previously (13, 14), with minor modifications. Plasmid pcDNA-HA-PML-IV (28) was kindly provided by C. Y. Choi (Sungkyunkwan University, Republic of Korea). Tat-expressing plasmids were constructed by cloning wild-type (wt) and mutant (mt) Tat

cDNAs (for sequences, see Table S1 in the supplemental material) into the pSV2-Tat (from J. Sodroski), pcDNA3-Flag (Invitrogen), pGEX-5X-1 (Amersham), or pRSETc (Promega) plasmid. Mutant Tats and shRNA/small interfering RNA (siRNA)-resistant silent mutant PKR/p53-expressing plasmids were generated using the QuikChange site-directed mutagenesis kit (Stratagene), and the PCR products were verified by sequencing. “Off-target effects” of each siRNA (si-PKR and si-p53, designed and provided by Invitrogen Stealth) and shRNA (sh-PKR) on HIV-1 replication in infected cells were evaluated with each siRNA/shRNA-resistant expressing plasmids (pcDNA-PKR/m and pcDNA-p53/m, having silent mutations on the target site of each siRNA/shRNA) (data not shown). PCR primers and siRNA/shRNA sequences used for the present study are summarized in Tables S2 and S3 in the supplemental material. Cells were transfected with these expression plasmids using Lipofectamine 2000 (Invitrogen) or by electroporation. Antibodies against p53 (DO-1; Santa Cruz Biotechnology), PKR and phospho-PKR (Cell Signaling), Tat (monoclonal Ab-N3 [Abcam]; rabbit antiserum [AIDS Research and Reference Reagent Program]), phospho-p53 (Cell Signaling), p21^{waf1/cip1} (Cell Signaling), and Puma (Cell Signaling), anti-Flag monoclonal antibody (Mab) (Cell Signaling), anti-cyclin T1 polyclonal antibody (PAB) (Cell Signaling), and antibodies against HIV-1 p24 (Abcam and Chemicon), β -actin (Sigma), and α -tubulin (Sigma) were used.

Recombinant Tat, eIF2 α , and PKR protein expression and purification. (i) **His-tagged HIV-1 Tat and human eIF2 α protein.** 6 \times His-tagged wild-type (wt) and mutant (mt) (Ala-A and Asp-D) Tat genes and wt and mt eukaryotic initiation factor 2 α subunit (eIF2 α) genes were cloned into the pRSETc/pcDNA/pSLX expression plasmid. *Escherichia coli* BL21 (Stratagene) was transformed with these plasmids and cultured in 2 \times YTA broth medium (50 μ g/ml ampicillin). Recombinant proteins were used for the experiments after purification.

(ii) **GST-Tat and GST-PKR fusion proteins.** Glutathione S-transferase (GST)-Tat and GST-PKR recombinant proteins (cloned in the pGEX-5X-1 vector) were prepared as described previously (29, 30), with minor modifications.

Yeast two-hybrid experiments. Yeast two-hybrid experiments were performed using the Matchmaker LexA two-Hybrid system (Clontech) as described previously (31). PKR was cloned into the DNA-binding domain (BD)-containing pLexA vector (His3 Amp^r), and HIV-1 *tat* or eIF2 α gene was cloned into the activation domain (AD)-containing pB42AD vector (Trp1 Amp^r) and then transformed into yeast strain EGY48. Positive clones were selected in UHW-auxotrophic minimal agar medium containing 2% glucose, and β -galactosidase (β -gal) expression was examined in UHW-auxotrophic medium supplemented with 2% galactose, 1% raffinose, 80 mg/liter X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and BU salt. Blue colonies indicate direct interactions between the two molecules *in vivo*.

In vitro kinase assay with recombinant proteins Tat, eIF2 α , and PKR. *In vitro* kinase assays were performed as described previously (14). Purified recombinant GST-PKR (0.2 μ g) was preactivated with poly(I-C) at 30°C for 1 h in the presence or absence of 1 μ Ci of [γ -³²P]ATP and then incubated with 0.5 μ g of 6 \times His(GST)-wt or -mt Tat or eIF2 α at 30°C for 1 h or for the time periods described in the figure legends. Each reaction was separated on a 12% or 15% SDS-polyacrylamide gel. Tat/eIF2 α phosphorylation was autoradiographed by exposing a dried gel to X-ray film (Eastman Kodak Co.) or by Western blot analysis using anti-phospho-Thr (Cell signaling) and/or anti-phospho-Ser (Zymed Co.) antibodies.

ESI-MS/MS analysis of PKR-treated Tat. Mass spectrometry (MS) was performed as described previously (14) with minor modifications. Tat bands following *in vitro* kinase reaction with PKR were gel extracted and digested with trypsin. The tryptic peptides were subjected to liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS) in a data-dependent scan mode. MS/MS spectra were searched via the Turbo SEQUEST algorithm against a target protein (HIV-1 Tat) database,

and the resulting identified phosphopeptides were further validated by manual inspection.

PKR-mediated Tat phosphorylation *in vivo*. Flag-tagged wt or mt Tat-expressing plasmids were transfected into PKR-normal (sh-con) or PKR^{KD} (sh-PKR) HEK293 cells or cotransfected with PKR-expressing plasmid (pcDNA-Myc-PKR) into HEK293 cells. Cells were cultured for 48 h, then treated with 50 nM calyculin A (Ser/Thr phosphatase inhibitor; Sigma) before harvest, and lysed with lysis buffer. Portions of cell extracts were treated with 1 U/ μ l of Lambda protein phosphatase (LPPase) (Sigma) for 1 h at 30°C. Tat phosphorylation was assessed by Western blotting using anti-Flag MAb, anti-phospho-Thr PAb (Cell Signaling), and anti-phospho Ser PAb (Zymed Co.).

HIV-1 replication and titration. The HIV-1 titer in culture supernatant was assessed with the Alliance HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (Perkin-Elmer Life Sciences). In brief, C8166 cells, human peripheral blood mononuclear cells (PBMCs), or primary CD4⁺ T cells were transfected with 1 μ g of wt or mt Tat-containing HXB2 cDNA by electroporation or infected with HIV-1 variants. Complement assay was performed by cotransfection with pcDNA-Flag-tat (wt or mt) and HXB2/ Δ Tat cDNA. After transfection/cotransfection/infection, the HIV-1 titers in the supernatants were assessed using the HIV-1 p24 ELISA kit, and intracellular HIV-1 was analyzed by Western blotting using anti-p24 antibody.

HIV-1 infection of human PBMCs and purified CD4⁺ T cells. Human peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers or human CD4⁺ T cells isolated from the human PBMCs using the Dynabeads Untouched human CD4 T cell kit (Invitrogen) were cultured for 3 days in RPMI 1640 supplemented with 0.25 μ g/ml phytohemagglutinin P (PHA-P), human interleukin 2 (hIL-2) (100 units/ml for CD4⁺ T cells) and 10% FBS before infection. After infection with the HIV-1_{IIIB} strain at a multiplicity of infection (MOI) of 0.1 to 1, cells were cultured in a medium supplemented with hIL-2 at a final concentration of 100 units/ml. Three to 4 days after infection, the HIV-1 titer in each culture supernatant was measured with the HIV-1 p24 ELISA kit (Perkin-Elmer). HIV-1 p24, p53, and PKR in the infected cells were assessed by Western blotting.

Reporter assays. The long terminal repeat (LTR)-conjugated luciferase reporter plasmid (pLTR-luc) was constructed by cloning the HIV-1_{IIIB} LTR sequence into the PGL-3 vector (Promega). Luciferase reporter assays were performed as described previously (13) with minor modifications. To assess the effects of p53 and PKR on Tat activity, cells were transfected with 200 ng of pLTR-luc and 100 ng of pcDNA-Flag-tat (or pSV2-tat) together with different amounts of pcDNA-p53 and/or pcDNA-PKR plasmids using Lipofectamine 2000 (Invitrogen). In the reporter assay, pCMV-lacZ (20 ng) was always cotransfected as a transfection control. At 48 h after transfection, the luciferase activity of each sample was measured and normalized to the β -galactosidase activity of each transfection control (20 ng of pCMV-lacZ) using the Bright-Glo luciferase assay system (Promega) and Genios luminometer (Tecan, Austria). Data are represented as means \pm standard deviations (SD). The LTR-chloramphenicol acetyltransferase (CAT) reporter system (pU3III-CAT plasmid) was obtained from the ARRRP (NIH, USA). The CAT reporter assay was performed as described previously (27).

RNA retardation assays. TAT-TAR interaction and retardation assays were performed as described previously (32) with some modifications. ³²P-labeled wt TAR RNA (or mutant TAR having 20-GACUCGA-26 mutations at the bulge region) was synthesized by *in vitro* transcription of pTZ18R-TAR using a commercial T7 RNA polymerase system (NEB) and [α -³²P]UTP (Amersham). Phosphorylated Tat protein was prepared by incubating Tat protein with preactivated PKR for the indicated period of time (0 to 120 min) in the presence or absence of [γ -³²P]ATP. Tat protein was incubated with ³²P-labeled TAR RNA for 15 min in 10 μ l of RNA binding buffer (15 mM HEPES-KOH [pH 7.4], 5 mM MgCl₂, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 10 μ g/ml aprotinin, 1 μ M dithiothreitol [DTT], 1 unit of RNasin [Promega]). The TAT-TAR binding assay was

also performed with different concentrations of wild-type or phosphor-mimic (D-mt) Tat proteins and 3 pmol of ³²P-labeled TAR RNA. The retardation assay was carried out on a 3% native or denaturing (SDS) polyacrylamide gel and visualized by autoradiography.

Immunocytochemistry analyses. Immunocytochemistry was performed as described previously (13) with minor modifications. Cells were transfected with appropriate expression plasmids or treated with recombinant Tat proteins and then fixed and permeabilized with Cytofix/Cytoperm (BD Bioscience Inc.). Cells were then incubated for 1 h with primary anti-Flag (1/500), antihemagglutinin (anti-HA) (1/500), or anti-Tat antibodies and then incubated with fluorescence (fluorescein isothiocyanate [FITC] or Texas Red)-labeled secondary antibodies (1/500) overnight at room temperature. Fluorescence signals were observed on a fluorescence microscope (Olympus X100) or confocal laser scanning microscope (Zeiss F510).

Co-IP assays. Coimmunoprecipitation (co-IP) assays were performed as described previously (14) with minor modifications. C8166 cells were transfected with wt or mt Tat-expressing plasmids (pcDNA3-Flag-tat) using Lipofectamine 2000 (Invitrogen). After 24 h, Tat in cell lysates was immunoprecipitated with anti-Flag antibody (M2; Sigma) together with protein A/G agarose beads (Santa Cruz) at 4°C for 5 h. Pellets were washed and assessed by Western blotting. Co-IP of cyclin T1 (CycT1) and Tat was performed as follows. 6 \times His-Tat was fully phosphorylated by overnight incubation with preactivated PKR in the presence of [γ -³²P]ATP and was incubated further with HeLa cell extracts at 4°C for 3 h. The mixtures were immunoprecipitated with anti-CycT1 antibody (Santa Cruz), and Tat was assessed by Western blotting and autoradiography.

GST pulldown experiments. Reaction mixtures containing GST-Tat (wt or mt) proteins were incubated with 1 mg of HeLa cell nuclear extracts at 4°C for 4 h. Reaction mixtures were incubated for 30 min with 10 μ l of glutathione beads (Amersham-Pharmacia Biotech), washed three times with binding buffer, and analyzed by Western blotting assay.

Statistical analysis. Statistical analysis was performed using Student's *t* test with GraphPad Instat software. A *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. NCBI GenBank accession numbers for the major genes and proteins that are mentioned in the text are as follows: p53, [XM_008679.2](#); PKR, [NM_002759.3](#); HIV-1 Tat, the sequence and accession number of each variant mentioned in the manuscript are summarized separately in Table S4 in the supplemental material; eIF2 α , [J02645.1](#); TAR, [M36464.1](#).

RESULTS

HIV-1 infection activates p53, which inhibits Tat function. HIV-1 infection increased the levels of p53 in primary peripheral blood mononuclear cells (PBMCs) and purified CD4⁺ T cells (Fig. 1A). Treatment of HIV-1-infected cells with pifithrin- α (Pft- α , a p53 inhibitor) significantly increased viral replication after infection (Fig. 1B), implying that induced p53 suppresses HIV-1 replication in infected cells. In addition, induced p53 after infection led to the expression of several p53 target genes, including p21, Puma, and PKR, and the expression profile of p53 target genes in primary CD4⁺ T cells was very similar to that in the C8166 human T cell line (Fig. 1C), indicating that C8166 cells could be substituted for primary CD4⁺ T cells in the following experiments. HIV-1 replication in C8166 cells was markedly enhanced by p53-knockdown (p53^{KD}) in HIV-1-infected cells (Fig. 1D). It was reported that HIV-1 infection induces the expression of type 1 interferon (IFN), accompanied by upregulation of p53 mRNA in CD4⁺ T cells (10). To examine the effects of type 1 IFN on p53-mediated HIV-1 suppression, HIV-1 infected p53-normal (p53^{+/+}) and p53^{KD} cells were cultured in the presence of IFN- α / β neutralizing antibodies. As shown in Fig. 1E, the titer of virus was enhanced by blocking type 1 IFN in p53-normal cells, but the

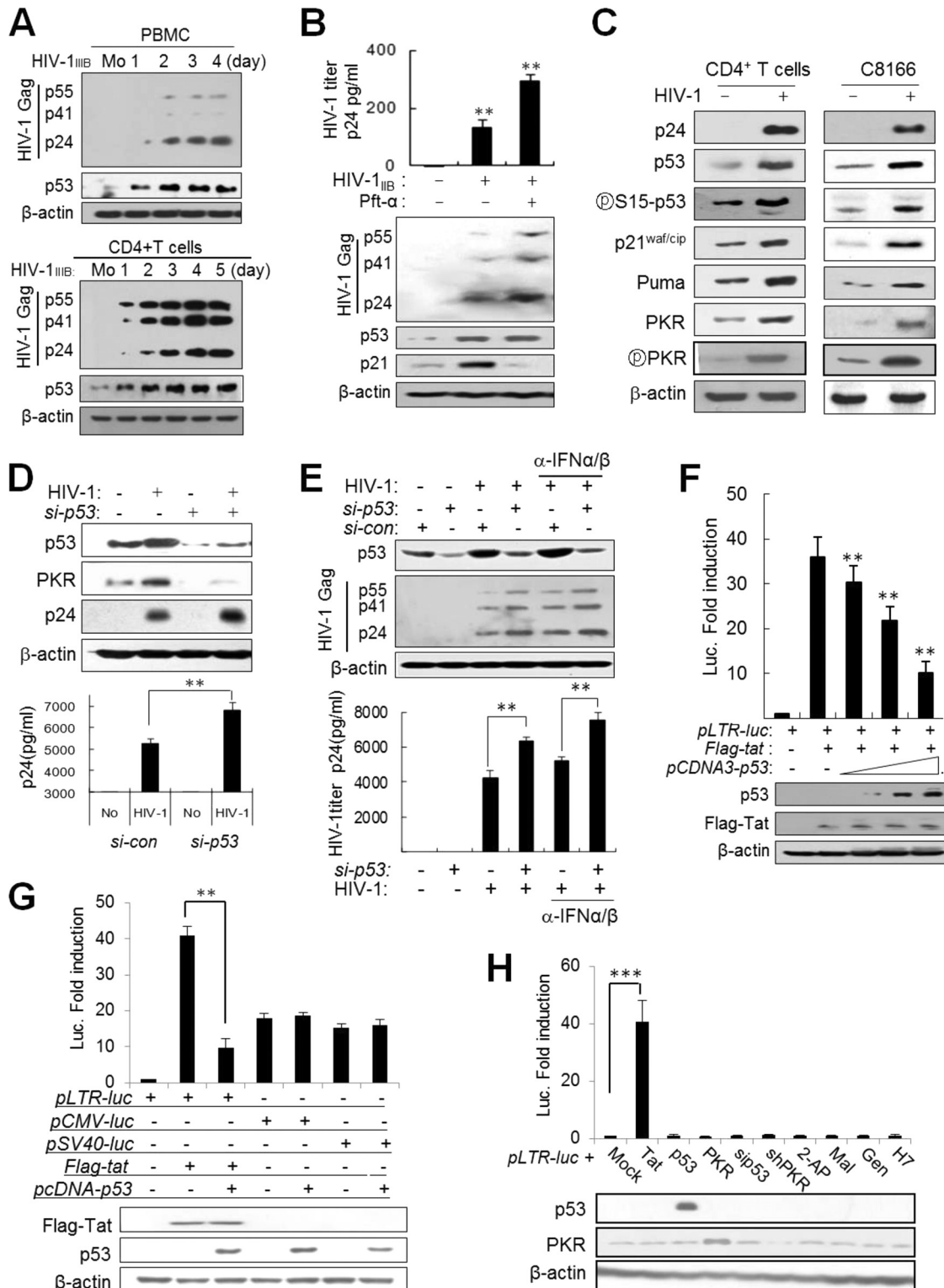


FIG 1 HIV-1 infection induces the expression of p53, and p53 inhibits HIV-1 replication by suppression of Tat activity through PKR. (A) Preactivated PBMCs (upper panel) and CD4⁺ T cells (lower panel) were infected with HIV-1_{IIIB} at an MOI of 1 for the indicated periods of time, and the levels of p53 and HIV-1 Gag proteins in infected cells were assessed by Western blotting with anti-p53 and anti-p24 antibodies. Mo, mock infection. (B) Upper panel, HIV-1-infected PBMCs were cultured in the presence or absence of 10 μ M pifithrin- α (Pft- α) (a p53 inhibitor). Four days after infection, titers of virus in culture supernatants were assessed by p24 ELISA and are represented as means \pm SD ($n = 3$). **, $P < 0.01$. Lower panel, HIV-1 Gag proteins in infected cells were assessed by Western blotting. (C) The expression of p53 and p53 target genes was assessed 4 days after infection of CD4⁺ T cells with HIV-1_{IIIB} and 40 h postinfection of C8166 cells with HIV-1 (HXBc2) at an MOI of 1. (D) HIV-1 replication was assessed in p53-normal and p53^{KD} (si-p53) (p53 siRNA; Cell Signaling) C8166 cells 48 h after infection (MOI of 1) from cell extracts by Western blotting (upper panel) and from culture supernatants using a p24 ELISA kit (represented as means \pm SD;

enhancement by p53 knockdown was more significant than that by blocking of type 1 IFN (Fig. 1E). These data indicate that p53 induced by HIV-1 infection plays a crucial role in the inhibition of HIV-1 replication in the infected cells. In an HIV-1 long terminal repeat (LTR)-luciferase (LTR-luc) reporter assay, ectopic expression of p53 inhibited Tat activity in a dose-dependent manner with little effect on Tat expression in p53^{-/-} cells (Fig. 1F). In order to assess whether p53-mediated Tat suppression is specific to the HIV-1 LTR promoter, other viral promoters were examined under the same conditions. The cytomegalovirus (CMV) promoter and the simian virus 40 (SV40) promoter were minimally affected by ectopic expression of p53 in luciferase reporter assays (Fig. 1G). Thus, p53-mediated Tat inhibition appeared to be due to suppression of Tat activity rather than to p53-derived general inhibition. The LTR-luc reporter alone without Tat was activated neither by ectopic expression of p53 and PKR nor by transfection of cells with siRNA or treatment of cells with kinase inhibitors (Fig. 1H), indicating that LTR-promoter activation was restricted only to Tat without being affected by the experimental procedures. We next examined whether p53 interacts directly with Tat protein. Direct interaction of p53 and Tat has been controversial (33–35), and we could not find any evidence of a direct interaction between p53 and Tat in coimmunoprecipitation (co-IP) assays or yeast two-hybrid analysis (data not shown). These data suggested that another factor(s) may be involved in p53-mediated Tat suppression rather than direct interactions between the two molecules.

PKR plays a crucial role in p53-mediated Tat suppression.

Next we investigated which factors are involved in p53-mediated Tat suppression. We found that a recombinant Tat protein was phosphorylated by p53-expressing cell lysates in a dose-dependent manner (Fig. 2A). We examined which protein kinases downstream of p53 were involved in Tat phosphorylation and inactivation. Among several kinase inhibitors, 2-aminopurine (2-AP) (a potent PKR inhibitor) restored Tat function from the p53-mediated Tat suppression in LTR-luciferase reporter assays (Fig. 2B), suggesting that PKR is likely involved in p53-mediated Tat suppression. Although 2-AP is a well-established PKR inhibitor, its nonspecific inhibition cannot be completely excluded. Therefore, PKR involvement was further verified by significant attenuation of p53-mediated Tat suppression upon PKR silencing (Fig. 2C). In addition, ectopic overexpression of PKR suppressed Tat function in a dose-dependent manner in p53^{-/-} cells (Fig. 2D). To examine the association of PKR-mediated Tat suppression with eIF2 α phosphorylation, PKR or p53 was cotransfected to Jurkat/eIF2 α ^{CA} cells (bearing eIF2 α -S51A, a constitutively active form of eIF2 α) with pSV2-Tat72. As shown in Fig. 2E, p53/PKR-

mediated Tat suppression was not attenuated in eIF2 α ^{CA} cells, suggesting that p53/PKR-mediated Tat suppression is not associated with the canonical PKR/eIF2 α translational inhibition pathway. These results were similarly repeated in human primary CD4⁺ T cells isolated from human PBMCs. HIV-1_{IIIIB} replication was significantly enhanced in CD4⁺ T cells when infected cells were treated with 2-AP (Fig. 2F). Taken together, our results imply that p53-mediated HIV-1 suppression is likely attributable to the p53/PKR-mediated Tat inactivation after HIV-1 infection.

PKR interacts with Tat and phosphorylates Ser/Thr residues on the first exon of Tat. PKR directly interacted with HIV-1 Tat in yeast two-hybrid and co-IP assays (Fig. 3A). In an *in vitro* kinase assay, Tat was more strongly phosphorylated by PKR than eIF2 α (Fig. 3B). Full-length Tat and the first exon part of Tat were phosphorylated to a similar extent by PKR, while the second exon part of Tat alone was not phosphorylated at all under the same conditions (Fig. 3C). The kinetics of Tat phosphorylation by PKR revealed that the Tat band shifted up to multiple bands over time during *in vitro* kinase assays with recombinant PKR and Tat protein (Fig. 3D), suggesting that Tat is likely to have multiple PKR phosphorylation sites. The Tat bands were clearly shifted in PKR-normal cells, while these shifted band patterns were not observed in PKR^{KD} (sh-PKR) cells (Fig. 3E). Furthermore, the shifted Tat bands in PKR-normal cells disappeared upon additional treatment with phosphatase (λPPase) (Fig. 3E). The intensity of the shifted Tat band increased upon ectopic expression of PKR (Fig. 3F). These data indicated that the shifted Tat bands were attributable to Tat phosphorylation by PKR. Phosphorylation of Tat by PKR on residues 62, 64, and 68 was previously reported in an *in vitro* biochemical study (24). In the present study, however, S62A-T64A-S68A mutant (mt) Tat was also equivalently phosphorylated by PKR (Fig. 3G, top), indicating that S62-T64-S68 are not the major sites on Tat for phosphorylation by PKR.

PKR phosphorylates Tat on five Ser/Thr residues, mainly at T23, T40, and S46. To determine the target sites for Tat phosphorylation by PKR, we constructed Tat mutants with Ala substitutions at nine Ser/Thr residues and performed *in vitro* kinase assays with PKR. Tat phosphorylation by PKR was markedly attenuated by the substitution T23A, T40A, or S46A, weakly attenuated by S62A or S68A, and not attenuated at all by T64A compared with wt Tat (Fig. 4A). When the five Ser/Thr residues in the first exon were replaced by Ala, not only the first exon (Tat72; Fig. 4B, right panels) but also full-length Tats (Tat86; Fig. 4B, left panels) were not phosphorylated by PKR, suggesting that PKR phosphorylates exclusively the 5 Ser/Thr sites in the first exon of Tat. These 5 phosphorylation sites on the Tat functional motif (Fig. 4C) were found to be highly conserved in HIV-1 strains such as Ba-L, Bru,

n = 3) (lower panel). **, *P* < 0.01 for untreated versus treated cells. (E) Effects of p53 and type 1 interferon (IFN) on HIV-1 replication and PKR expression. Upper panel, p53-normal (si-con) and p53^{KD} (si-p53) C8166 cells were infected with HIV-1 (HXB2) at an MOI of 1 in the presence or absence of 5 μg/ml anti-IFN- α /β neutralizing antibodies (BioLegend Inc.). After 48 h, cell extracts were assessed by Western blotting with anti-HIV-1 Gag antibodies. Lower panel, the titers of HIV-1 in culture supernatants were assessed in triplicates at 48 h postinfection using a p24 ELISA kit (Perkin-Elmer) and are represented as means ± SD (*n* = 3). **, *P* < 0.01 versus control siRNA (si-con). (F) HCT116 p53^{-/-} cells were transfected with pLTR-luc (200 ng), pcDNA-Flag-tat (100 ng), and increasing amounts of pcDNA3-p53 (1, 2.5, and 5 μg), together with 20 ng of pCMV-lacZ as a transfection control. Luciferase activity was assessed 2 days after transfection and expressed after normalization to β-galactosidase activity. Data are represented as means ± SD (*n* = 4). Protein levels were assessed by Western blotting after normalization to β-actin. **, *P* < 0.01. (G) pLTR-luc, pCMV-luc, and pSV40-luc reporter plasmids were cotransfected with the indicated Tat or p53 plasmid combinations into HeLa cells together with pLTR-luc as a transfection control. Luciferase activity was expressed after normalization with the β-galactosidase activity of each reaction. Protein levels were assessed by Western blotting after normalization with β-actin. **, *P* < 0.01. (H) HeLa cells were cotransfected with pLTR-luc and other expression vectors together with pCMV-lacZ as a transfection control. In addition, HeLa cells transfected with pLTR-luc and pCMV-lacZ were treated with each kinase inhibitor as shown. Luciferase activity was assessed. ***, *P* < 0.001.

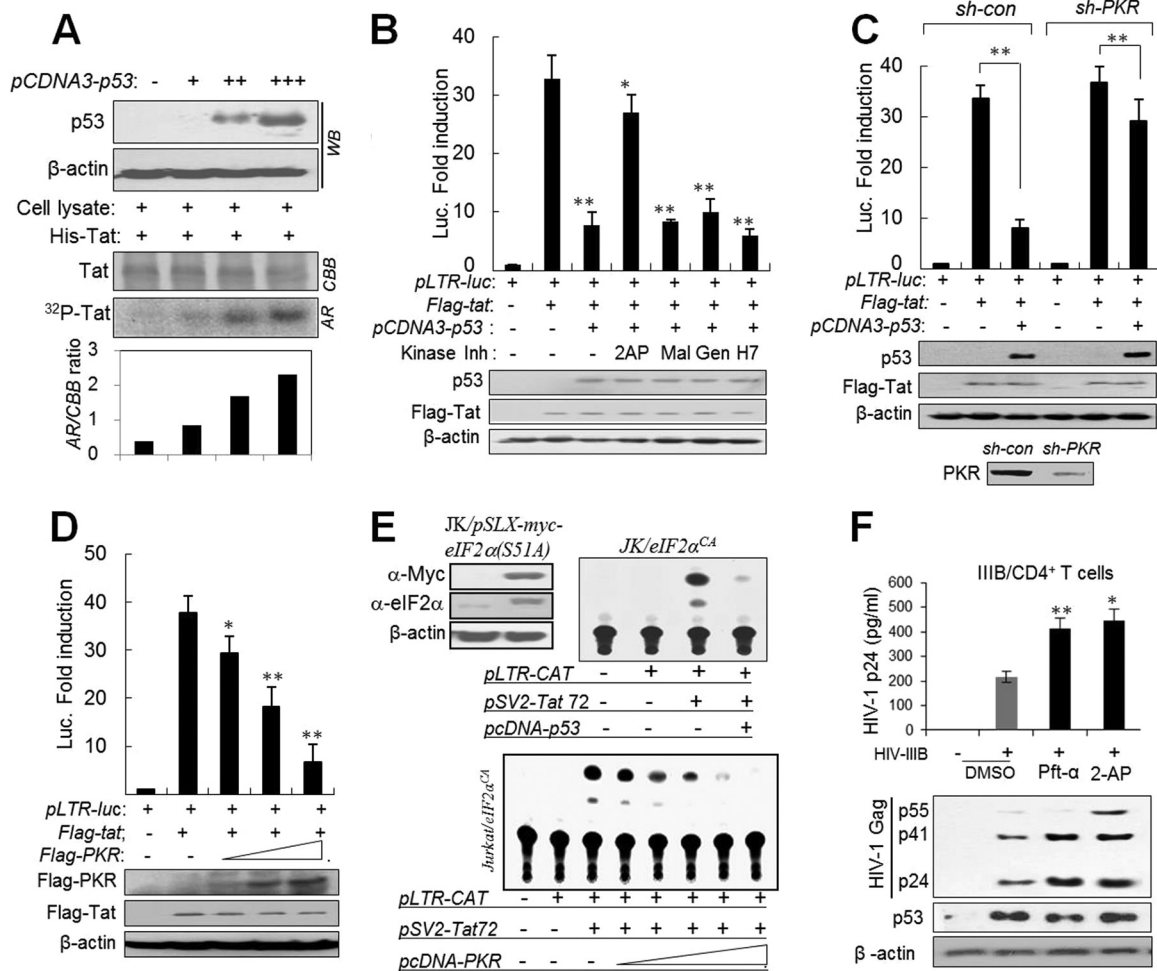


FIG 2 PKR plays a crucial role in p53-mediated Tat suppression. (A) p53-expressing cell lysates phosphorylate HIV-1 Tat. HCT116 p53^{-/-} cells were transfected with increasing amounts of pCDNA-p53 (1, 3, and 5 μg). Two days after transfection, p53 expression was assessed by Western blotting (upper panel), and the cell lysates were incubated with recombinant Tat proteins (6×His-Tat) in the presence of [γ -³²P]ATP. The reaction mixtures were immunoprecipitated with anti-His MAb and then applied to an SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue and autoradiographed (lower panel). (B) HCT116 p53^{-/-} cells were transfected with pLTR-luc (200 ng), pCDNA-Flag-tat (100 ng), and pCDNA3-p53 (5 μg), together with 20 ng of pCMV-lacZ as a transfection control. One day after transfection, cells were treated with the protein kinase inhibitor 2-AP (2 mM 2-aminopurine; PKR inhibitor), Mal (30 μM Mallotoxin; PKC inhibitor), Gen (20 μM Genistein; PTK inhibitor), or H7 (5 μM H-7; PKA and PKC inhibitor). After 24 h, luciferase reporter assays were performed. Values are means \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$. (C) PKR-normal (sh-con) and PKR^{KD} (sh-PKR) HCT116 p53^{-/-} cells were transfected as described above, and luciferase activity was assessed. Values are means \pm SD ($n = 4$). **, $P < 0.01$. (D) HCT116 p53^{-/-} cells were transfected with increasing concentrations of pCDNA-Flag-PKR, and luciferase activity was assessed. Values are means \pm SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$. (E) Ectopic expression of p53 and PKR inhibits Tat activity in eIF2α^{CA} (pSLX-myc-eIF2α-S51A-transformed) Jurkat cells (JK/eIF2α^{CA}). Myc-eIF2α expression was examined in JK/eIF2α^{CA} cells by Western blotting (upper left). JK/eIF2α^{CA} cells were transfected with pLTR-CAT and pSV2-tat72 together with pCDNA-p53 plasmids as indicated (upper right). JK/eIF2α^{CA} cells were cotransfected with pLRT-CAT and pSV2-Tat72 together with increasing concentrations of pCDNA-PKR plasmid (lower). Intracellular CAT activity was assessed 2 days after transfection. (F) Human CD4⁺ T cells purified from the PBMCs were infected with HIV-1_{IIIB} at an MOI of 1 for 1 h and then cultured in the presence or absence of 10 μM Pft-α or 2 mM 2-AP (PKR inhibitor) for 4 days. HIV-1 titer in the culture supernatants (upper panel) and intracellular Gag proteins (lower panel) were assessed by p24 ELISA and Western blotting, respectively, and results are represented as means \pm SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (untreated versus treated cells). All luciferase data in panels B to G were normalized to the β-galactosidase activity of each transfection control (20 ng of pCMV-lacZ), and expression levels of Tat, p53, and PKR were examined by Western blotting.

Oyi, JR, and IIIB, which are predominant in the United States and Europe (Fig. 4D; see Table S4 in the supplemental material). In order to demonstrate intracellular phosphorylation of Tat on these Ser/Thr sites, we tried to generate site-specific phospho-Tat antibodies, but our attempts were unsuccessful in several trials. Instead, we used anti-phospho-Thr and anti-phospho-Ser antibodies after immunoprecipitation to assess intracellular Tat phosphorylation by PKR at these Ser or Thr sites. Intracellular Tat phosphorylation at Thr sites (T23 and T40) was markedly im-

paired by either T23A or T40A substitution (Fig. 4E, upper panel). Among the three PKR Ser target sites (S46, S62, and S68), Tat phosphorylation was significantly attenuated by the S46A substitution (Fig. 4E, lower panel) in PKR-normal cells. However, in PKR^{KD} (sh-PKR) cells, intracellular Tat phosphorylation was barely detected or undetectable by phospho-Ser/Thr antibodies (Fig. 4E). These data demonstrated the intracellular phosphorylation of Tat at specific Ser/Thr sites by PKR. However, we cannot exclude the possibility that another kinase(s) might be involved in

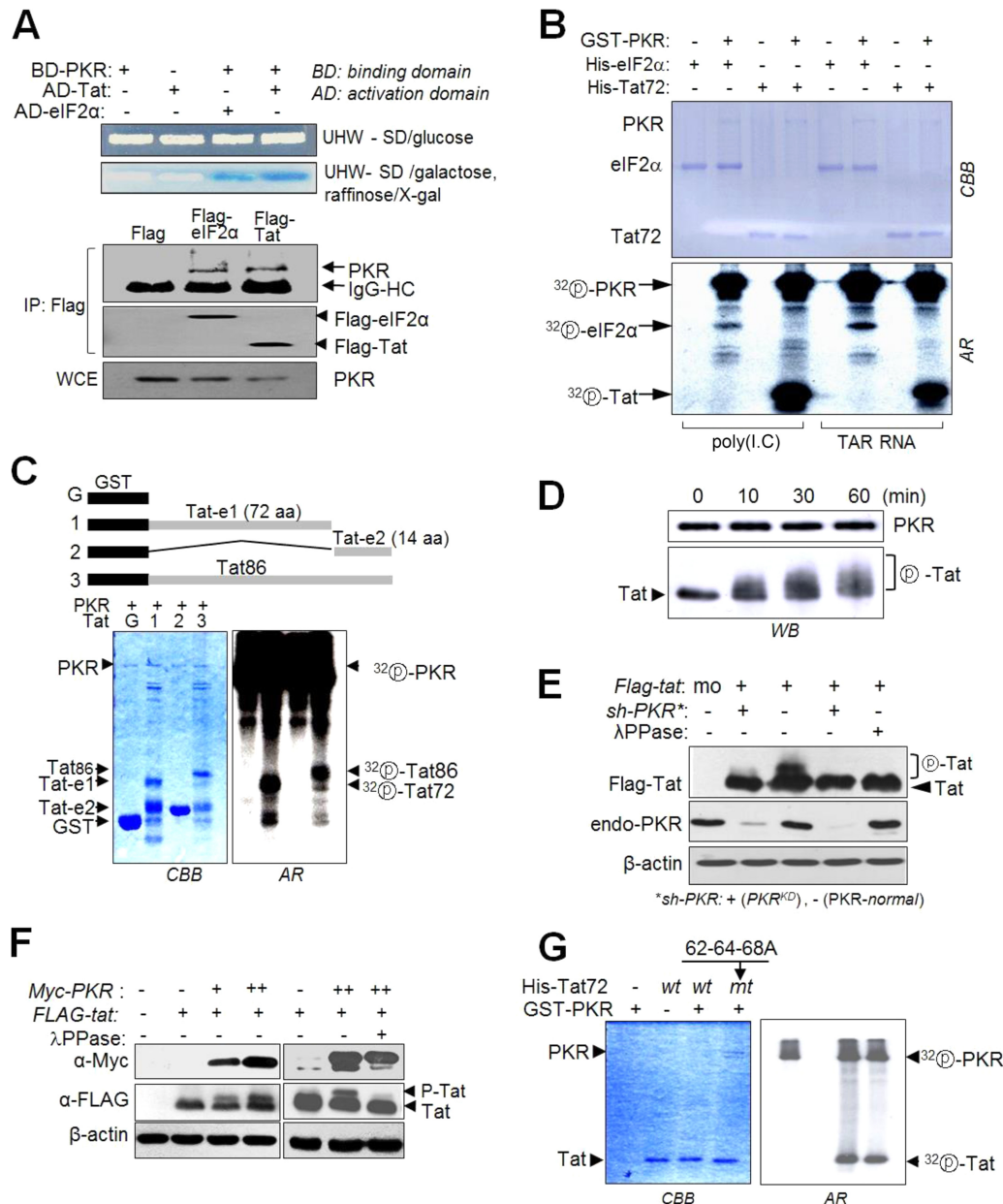
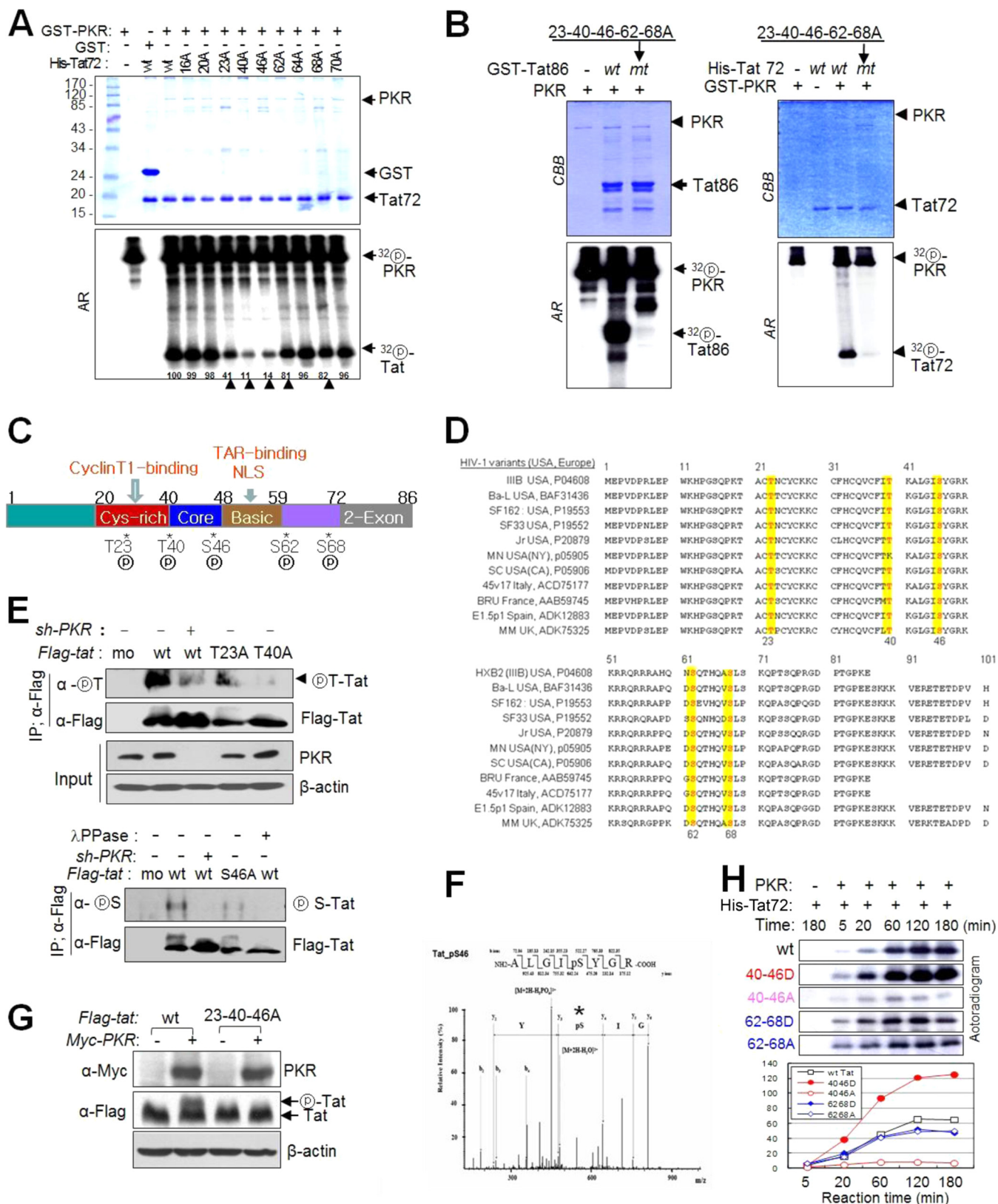


FIG 3 PKR phosphorylates the first exon of HIV-1 Tat. (A) Upper panel, yeast two-hybrid experiments with PKR and Tat as described in Materials and Methods. Lower panel, endogenous PKR was assessed by Co-IP with anti-Flag Ab in C8166 cells transfected with pcDNA-Flag-tat/eIF2α. (B) Recombinant GST-PKR protein (0.5 μg), preactivated with poly(I:C) or TAR RNA for 1 h, was incubated with 0.5 μg of recombinant His-eIF2α or His-Tat proteins in the presence of 1 μCi of [γ -³²P]ATP, and phosphorylation of Tat and eIF2α was examined by autoradiography after SDS-PAGE. (C) Recombinant GST-Tat proteins encoding the first exon (72 aa), second exon (14 aa), or full-length Tat (86 aa) were incubated with preactivated PKR in the presence of [γ -³²P]ATP, separated on a 12% polyacrylamide gel, and then assessed by autoradiography. (D) Preactivated PKR (GST-PKR) was incubated with column-purified His-Tat72 for 10 to 60 min at 30°C. Reaction mixtures were separated by 15% SDS-PAGE and then subjected to Western blot analysis. (E) PKR^{KD} (sh-PKR+) and PKR-normal (-) HEK293 cells were transfected with pcDNA3-Flag-tat. Cells were treated with 50 nM calyculin A (a phosphatase inhibitor) for 1 h prior to harvest, and then cell extracts were assessed by Western blotting. Parts of samples were additionally treated for 1 h with λPPase (1 U/μl) before SDS-PAGE. (F) PKR-mediated Tat phosphorylation *in vivo*. HEK293 cells were transfected with pcDNA-Flag-tat and different concentrations of pcDNA-Myc-PKR. Two days after transfection, cells were treated with 50 nM calyculin A for 1 h prior to harvest, and cell extracts were separated by 15% SDS-PAGE and then subjected to Western blot analysis with anti-Flag, anti-Myc, and anti-β-actin antibodies. One of the samples was treated with λPPase prior to the Western blot assay. (G) wt or 62-64-68A mt Tat (72 aa) was incubated with preactivated PKR in the presence of [γ -³²P]ATP. The reaction products were separated on a 12% SDS-polyacrylamide gel and then examined by autoradiography.

intracellular Tat phosphorylation, because a small amount of Tat phosphorylation was often detected even in the absence of PKR (sh-PKR), as shown in Fig. 4E (upper panel). S46 phosphorylation by PKR was further evidenced by ESI-MS/MS analysis (Fig. 4F). In

addition, the shifted Tat bands observed in wt Tat-expressing cells were not detected in 23-40-46A mt Tat-expressing cells (Fig. 4G). These data suggested that (i) PKR phosphorylated Tat predominantly at residues T23, T40, and/or S46 and (ii) T40-S46-phosphor-



ylation may affect subsequent phosphorylation events. This assumption was verified by *in vitro* kinase assays. The 40-46A mt Tat-72 (with T40A-S46A substitutions) was barely phosphorylated by PKR even though 3 remaining phosphorylation sites were intact, while 40-46D mt (Ser/Thr-to-Asp substitution, pseudophosphorylated form) was significantly phosphorylated by PKR over time, and the intensity of each band was much stronger than that of wt Tat-72 (Fig. 4H). In contrast, there was no difference in Tat phosphorylation between the 62-68A and 62-68D mutants, and band intensity was slightly attenuated compared to that of wt Tat (Fig. 4H). These data suggest that PKR-mediated Tat phosphorylation is a series of allosteric reactions (the first reaction facilitates the following reactions) starting from T40-S46 phosphorylation.

PKR-mediated Tat phosphorylation inhibits Tat activity. To examine the effects of Tat phosphorylation on Tat activity, we constructed recombinant Tat plasmids encoding single or multiple amino acid substitutions of Asp (D; phospho-mimic amino acid) and Ala (A; non-phospho amino acid) at the five PKR target Ser/Thr sites. Tat activity was significantly attenuated by D substitution at each Ser/Thr site except S62D, while the activities of corresponding A-mt Tats, except T23, were equivalent to or slightly higher than that of wt Tat (Fig. 5A). In addition, Tat activity was slightly attenuated by multiple A mutations (Fig. 5B), while being almost obliterated by multiple D mutations near the arginine-rich motif (ARM) region (46D, 4046D, 404662D, and 40466268D) (Fig. 5C). When the S23D mutation was added to the multiple D mutations around the ARM (234046D, 23404662D, and 2340466268D), Tat activity was completely abrogated (Fig. 5C), suggesting an additional important role for T23 in Tat activity. Tat T23 is highly conserved (over 90%) in HIV-1 strains that are prevalent in the United States and Europe but is uncommon (<4%) in African strains (Fig. 5D), most of which encode the natural T23N substitution (Fig. 5E). Our findings indicate that PKR-mediated Tat phosphorylation, at least within the five Ser/Thr residues of the first exon, suppresses Tat activity, followed by inhibition of HIV-1 replication after infection.

PKR-mediated Tat phosphorylation inhibits Tat nuclear localization. HIV-1 Tat is a nuclear protein, and the ARM (overlapped with the protein transduction domain [PTD]) is essential for Tat nuclear localization (36, 37) and plasma membrane transduction potential (38, 39). Residues T40, S46, S62, and S68 are adjacent to the ARM in the three-dimensional (3-D) structure of Tat (40). We examined the effects of PKR-mediated Tat phosphorylation on the membrane transduction potential and nuclear localization of Tat. When HeLa cell monolayers were incubated with phospho-free recombinant Tat protein in culture medium, Tat protein was observed mainly in the nucleus, but once fully phosphorylated by PKR *in vitro*, phospho-Tat protein resided

mostly in the cytoplasm after membrane transduction (Fig. 6A). When HeLa(LTR-luc) cells were treated, recombinant phospho-free normal Tat protein effectively induced expression of the luciferase gene in the nucleus in a dose-dependent manner, compared to ineffective induction by fully phosphorylated Tat protein (Fig. 6B). These data suggest that nuclear localization after membrane transduction of Tat protein was markedly impaired by PKR-mediated phosphorylation. Even though intracellular enhanced green fluorescent protein (EGFP) resides both in the cytoplasm and in the nucleus, ectopically expressed EGFP-Tat localized mainly to the nucleus. However, the nuclear localization of EGFP-Tat was inhibited by coexpression of p53 or PKR as shown by the distribution of control EGFP (Fig. 6C). This inhibitory phenomenon was also observed when cells were treated with IFN- α , which is a well-known PKR activator (Fig. 6C). In addition, phospho-shifted Tat bands were detected only in the cytoplasmic fraction of PKR-normal (sh-con) cells, whereas the majority of Tat protein in PKR^{KD} (sh-PKR) cells was detected in the nuclear fraction (Fig. 6D). Analysis of D-mt Tats to assess the effect of phosphorylation on the nuclear localization of Tat revealed that Tat nuclear localization was significantly impaired by D mutations at the PKR target Ser/Thr sites adjacent to the ARM (Fig. 6E). The intracellular distribution patterns of EGFP-Tat_{40-46-62-68D} and EGFP-Tat_{23-40-46-62-68D} were similar to that of control EGFP, while the T23D mutation alone did not affect nuclear localization of Tat (Fig. 6E), suggesting that Tat phosphorylation near the ARM might impair the nuclear localization signal of Tat, probably by disrupting the positive charge of the ARM. These results were further supported by analysis of the nuclear and cytoplasmic fractions. D-mt Tat proteins, particularly all-D-mt Tat, were detected mostly in the cytoplasmic fraction, whereas A-mt Tats were detected mainly in the nuclear fraction (Fig. 6F). These data indicate that the nuclear localization capacity of Tat was strongly impaired by PKR-mediated Tat phosphorylation, particularly adjacent to the ARM domain.

PKR-mediated Tat phosphorylation disrupts Tat binding to TAR RNA. The Tat-TAR interaction is essential for provirus transcription, and the ARM domain on Tat plays a crucial role in Tat binding to TAR RNA (36, 41). We performed electrophoretic mobility shift assay (EMSAs) with a ³²P-TAR probe and recombinant Tat protein at different molar ratios to determine the optimal concentrations for binding. ³²P-labeled TAR RNA was fully shifted by Tat at a 1:1 molar ratio for Tat (3 pmol) and TAR (3 pmol), while mutant TAR RNA was not shifted at all (Fig. 7A). When Tat was preincubated with different amounts of TAR RNA and then incubated with active PKR (PKRa) [preactivated with poly(I-C)], TAR-bound Tat was not phosphorylated by PKRa, while a molar excess of Tat unbound to TAR RNA was strongly phosphorylated (Fig. 7B), implying that once bound to TAR, Tat was protected from PKR-mediated phosphorylation. Conversely,

Tat86 (left) and Tat72 (right) proteins were incubated with preactivated PKR, Tat phosphorylation was assessed by autoradiography following SDS-PAGE. (C) PKR-mediated phosphorylation sites on the functional domain of HIV-1 (HXB2) Tat. (D) Amino acid sequences of the Tat proteins of typical HIV-1 variants in the United States and Europe (see Table S4 in the supplemental material). Five PKR target Ser/Thr sites are indicated by yellow boxes. (E) PKR-normal or PKR^{KD} HEK293 cells were transfected with Flag-tagged wt (86 aa) and single mt Tat plasmids. Cell extracts were immunoprecipitated with anti-Flag antibody and then assessed by Western blotting with phospho-Thr (upper panel)- and phospho-Ser (lower panel)-specific antibodies. (F) ESI-MS/MS of tryptic peptides of Tat. The assigned spectrum matched the given phosphopeptide sequence corresponding to residues 42 to 49 of HIV-1 Tat (ALGIpSYGR, *m/z*). *, phosphorylation site on the peptide. (G) HEK293T cells were cotransfected with Flag-tagged wt and 23-40-46A triple mt Tat86-expressing plasmids and PKR-expressing (Myc-PKR) plasmids. Samples were assessed by Western blotting with anti-Flag and anti-Myc antibodies. (H) His-tagged wt and double mt (S/T-to-D/A substitutions) Tat72 proteins were incubated with preactivated PKR (GST-PKR) for the indicated periods of time. Reaction products were assessed by autoradiography after SDS-PAGE (left). The relative band intensity of each sample is presented in a histogram (right).

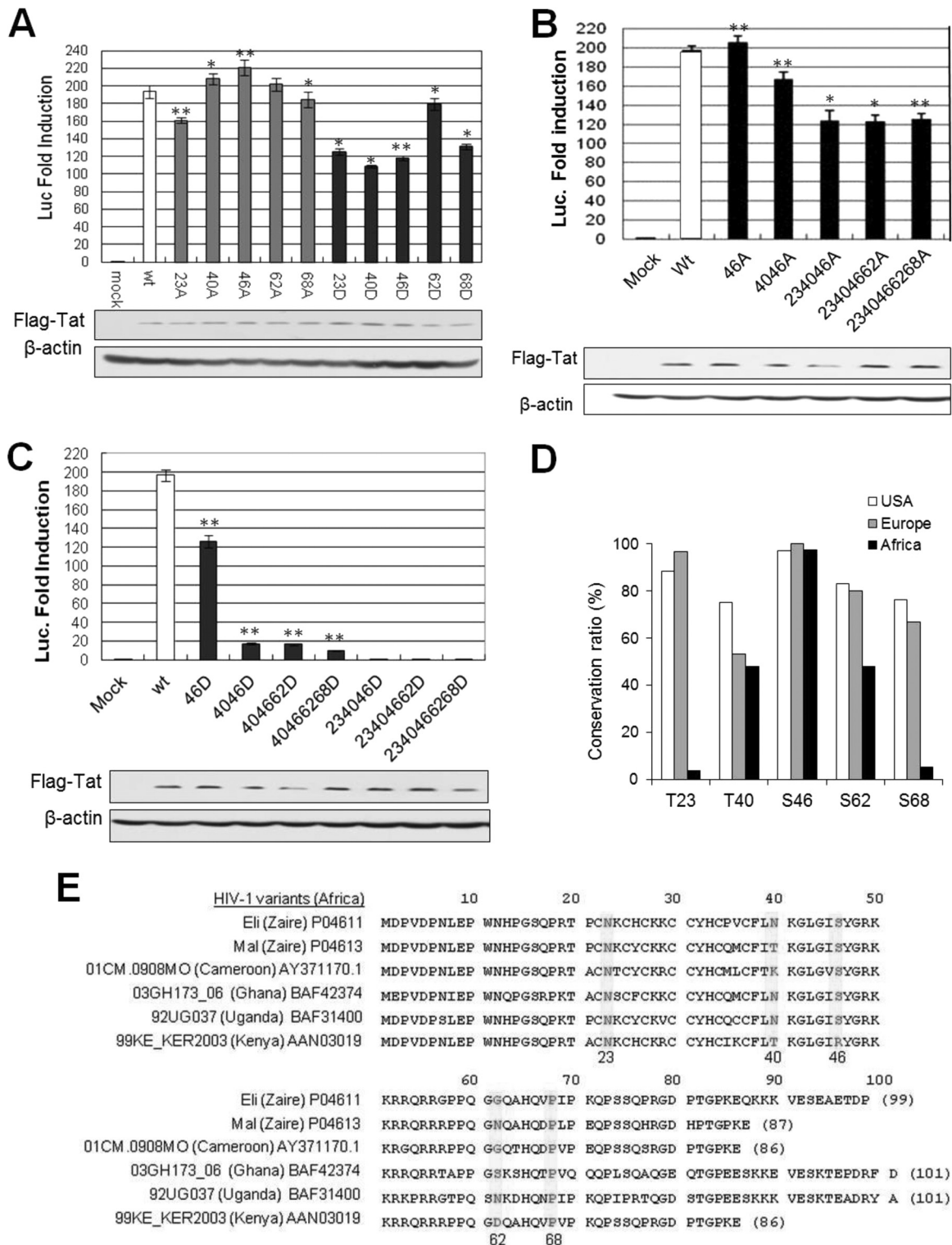


FIG 5 Effects of PKR-mediated Tat phosphorylation on Tat activity. (A) Single mt (Ser/Thr to non-phospho amino acid Ala [A] or phospho-mimic amino acid Asp [D]) Tat activity in luciferase reporter assay. HeLa cells were cotransfected with each Flag-tagged wt or mt Tat plasmid (100 ng) and pLTR-luc reporter plasmid (200 ng), with pCMV-LacZ (20 ng) as a transfection control. Two days after transfection, luciferase activity was assessed in triplicates, and fold activities normalized with the transfection control are represented as means \pm SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (mt versus wt Tat). (B and C) The activities of multiple A mutant (B) and D mutant (C) Tats were assessed by LTR-mediated luciferase reporter assay. (D) The sequence conservation ratio of each PKR target Ser/Thr site on HIV-1 Tat was assessed among 206 HIV-1 strains (101 strains from the United States, 30 from Europe, and 75 from Africa). Information on the 206 HIV-1 strains and their respective Tat amino acid sequences is shown in Table S4 in the supplemental material. (E) Alignment of HIV-1 Tat sequences of 6 African strains. Five PKR target sites, designated by yellow boxes, are mostly mutated with the non-phospho-form amino acids.

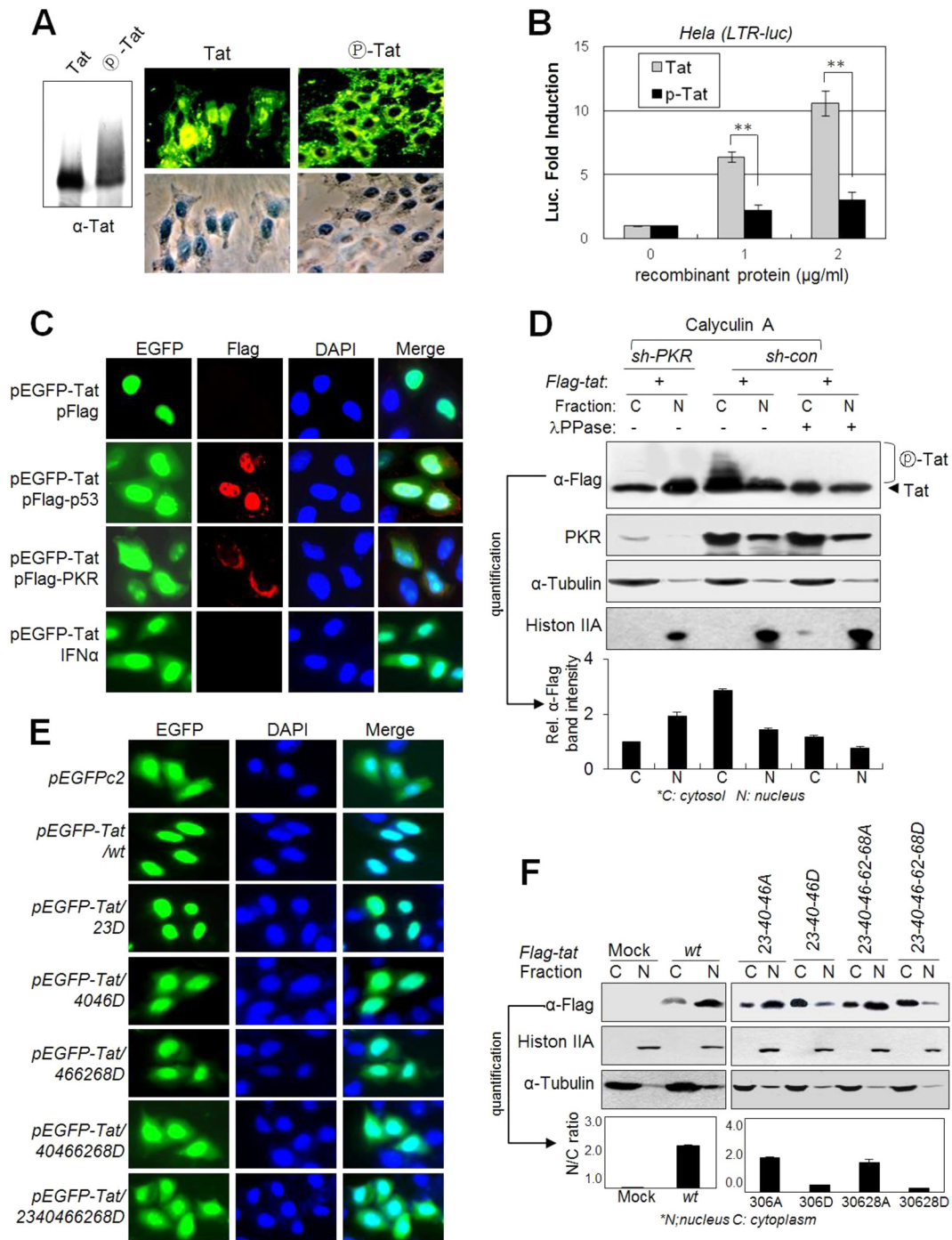


FIG 6 PKR-mediated Tat phosphorylation blocks Tat nuclear localization. (A) Phospho-free normal Tat and phospho-Tat (fully phosphorylated by PKR for 4 h) recombinant proteins were assessed by Western blotting (left panel) with anti-Tat Ab. HeLa cell monolayers were incubated with 1 μg/ml normal or fully phosphorylated Tat proteins in DMEM. After 1 h of incubation, cells were washed and gently trypsinized (1× trypsin-EDTA) for 2 min at 37°C to remove membrane-bound unpenetrated Tat protein. Intracellular Tat was assessed by immunocytochemistry with anti-Tat and FITC-conjugated secondary antibodies. (B) HeLa cells transfected with pLTR-luc (200 ng) and pCMV-lacZ (20 ng) plasmids were treated with increasing amounts of normal Tat and phospho-Tat proteins (0 to 2 μg/ml). After 24 h, luciferase activity was assessed and is represented as means ± SD ($n = 3$). *, $P < 0.01$. (C) pEGFP-tat-transfected HeLa cells were cotransfected with pcDNA3-Flag-p53 or pcDNA3-Flag-PKR or treated with 1,000 U/ml of IFN-α. Twenty-four hours after transfection, cells were photographed under a fluorescence microscope (magnification, ×400). (D) PKR-normal (sh-con) and PKR^{KD} (sh-PKR) HEK293 cells were transfected with pFlag-tat. Two days after transfection, cells were treated with 50 nM calyculin A for 1 h prior to harvest, and then nuclear and cytosolic fractions from the cell extracts were prepared using the NER/PER kit (Pierce) and assessed by Western blotting. Shifted Tat bands are indicated. Relative intensities of Tat bands in the Western blot are represented as means ± SEM ($n = 3$) after quantification three times using TINA 2.0 software (Raytest, Straubenhardt, Germany). (E) HeLa cells were transfected with GFP-conjugated wt or D-mt Tat plasmids. Two days after transfection, cells were fixed and photographed under a fluorescence microscope (magnification, ×200). (F) HEK293 cells were transfected with Flag-tagged wt or mt Tat plasmids as indicated. Nuclear and cytosolic fractions from the cell extracts were assessed by Western blotting with appropriate antibodies. Relative intensities of the Tat bands in the cytoplasm and the nucleus are represented.

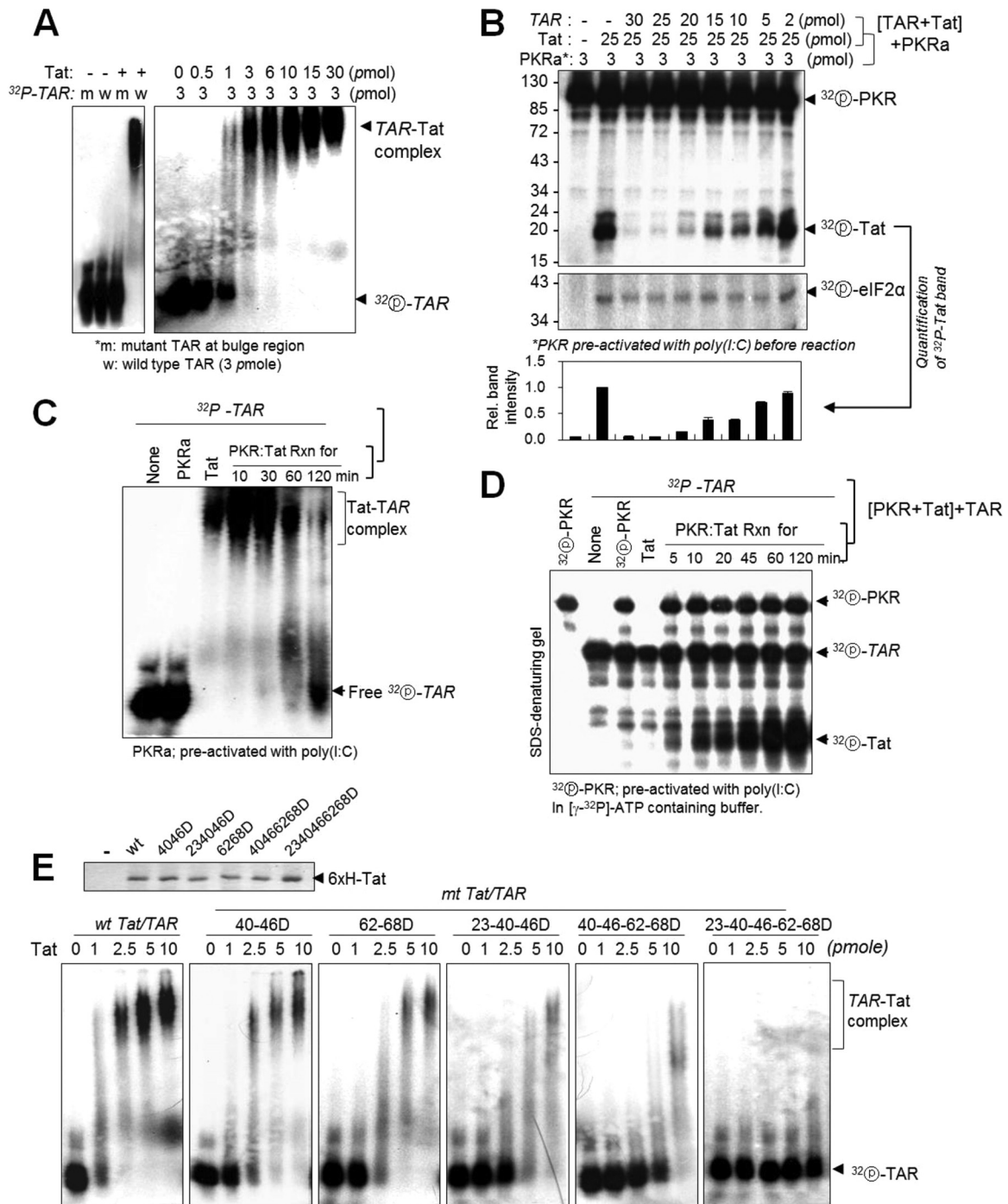


FIG 7 PKR-mediated Tat phosphorylations disrupt Tat binding to TAR RNA. Tat-TAR interaction was assessed by RNA retardation assay as described in Materials and Methods. (A) Left panel, ³²P-labeled wild type (w) and mutant (m) (22-UCU-24 to CUC at bulge region) TAR RNAs (3 pmol) were incubated with 3 pmol of Tat protein on ice for 15 min, separated on a 3% native polyacrylamide gel, and autoradiographed. Right panel, increasing amounts of Tat protein were incubated with 3 pmol of ³²P-labeled wt TAR RNA and then assessed as described above. (B) Tat protein (25 pmol) was preincubated with different amounts of TAR RNA, and the reaction mixtures were further incubated with PKR preactivated with poly(I:C) in a kinase reaction buffer containing [γ -³²P]ATP. The reaction products were separated on a 12% SDS-polyacrylamide gel and assessed by autoradiography. Relative intensities of phosphor-Tat bands are represented as means \pm SD ($n = 3$). (C and D) Tat protein (15 pmol) was incubated with preactivated PKR (3.5 pmol) in the presence of cold ATP (C) or [γ -³²P]ATP (D) for the indicated time periods. The reaction mixtures were further incubated with 3 pmol of ³²P-TAR RNA on ice for 15 min, separated on 3% native (C) or 12% denaturing (D) polyacrylamide gels, and analyzed by autoradiography. (E) Tat (wt and mt) proteins were fully normalized (upper panel), and then the indicated amounts of wt and mt Tat proteins were incubated with 3 pmol of ³²P-labeled TAR RNA, separated on a 3% native polyacrylamide gel, and assessed by autoradiography.

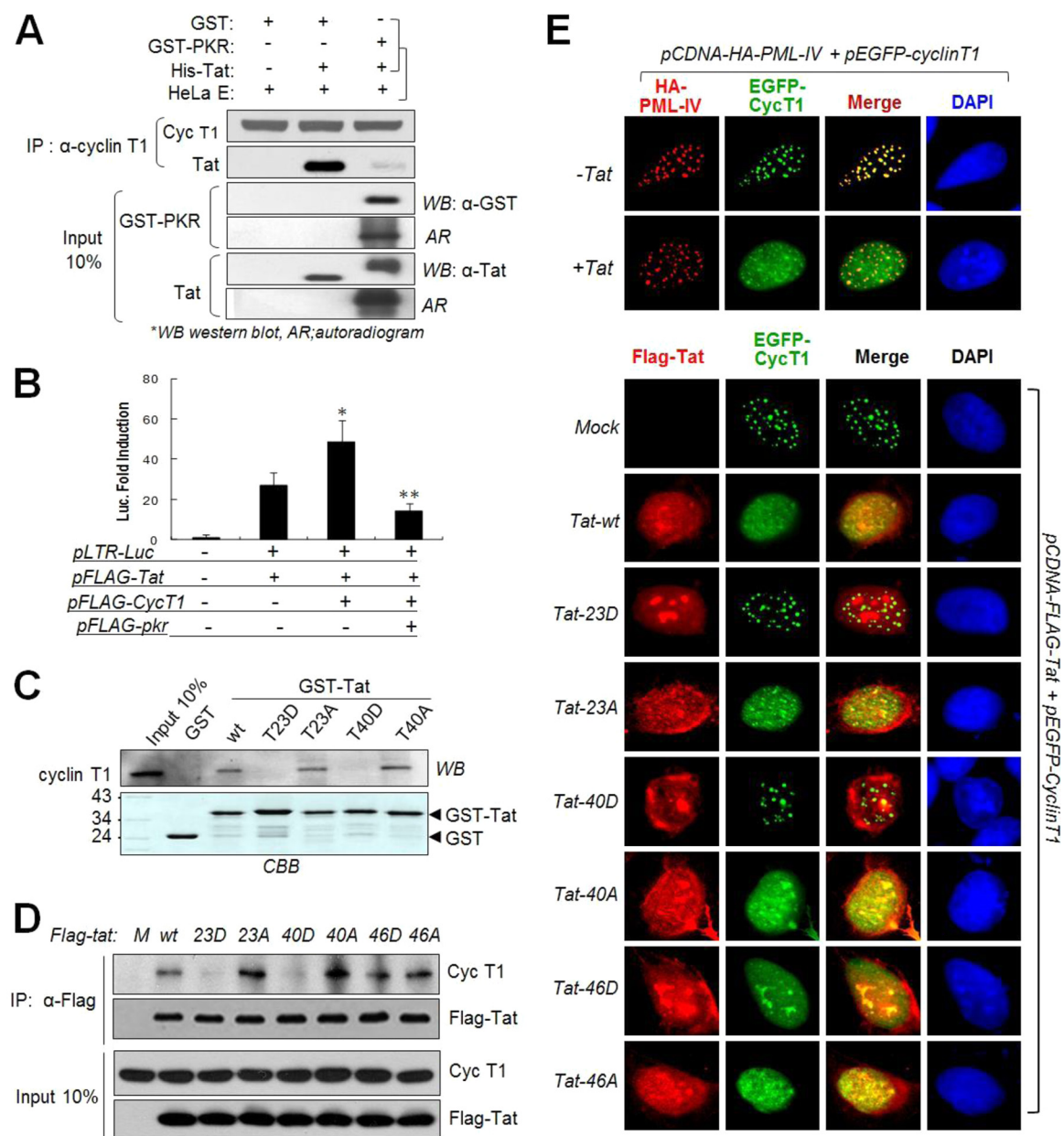
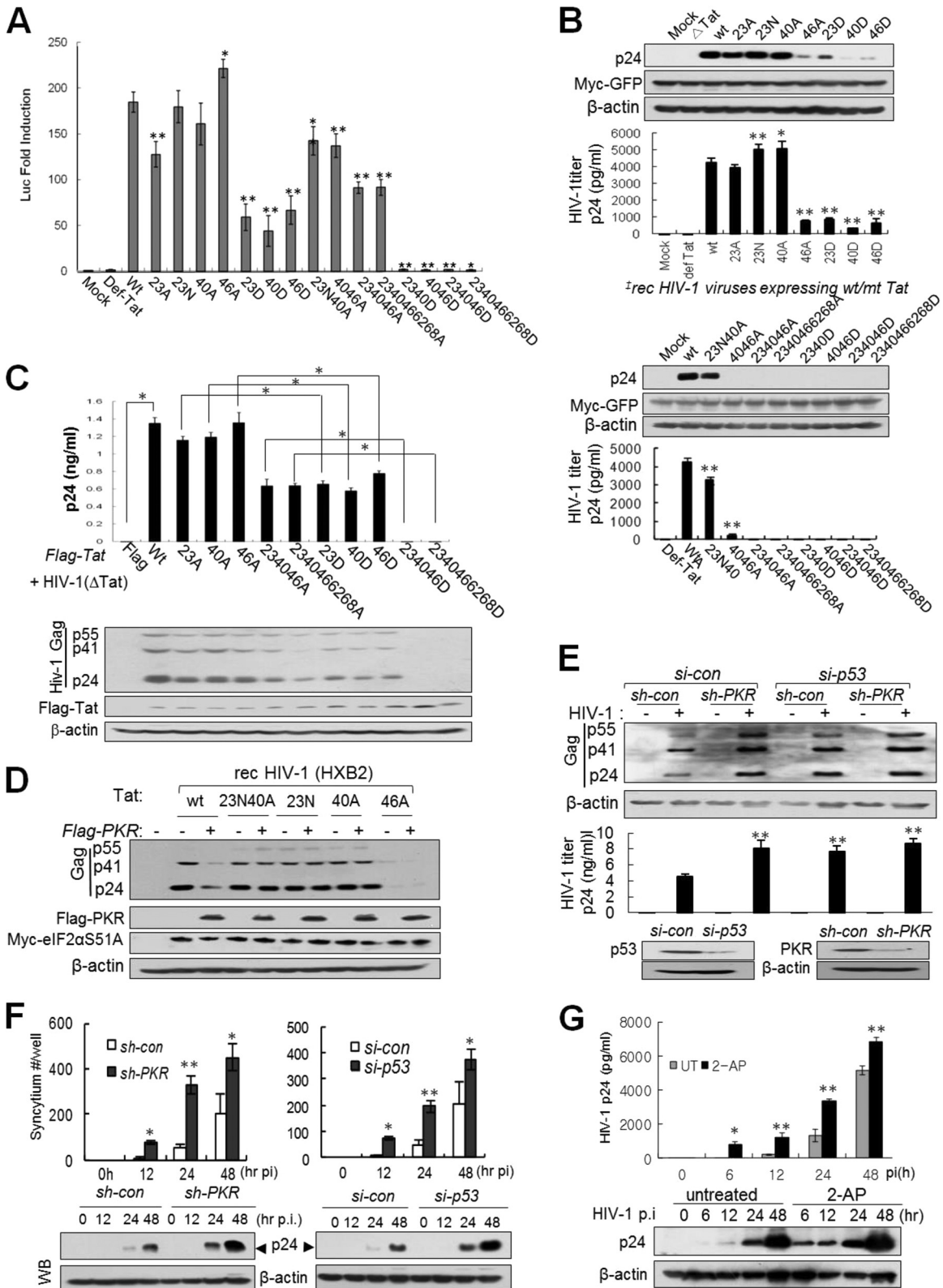


FIG 8 PKR-mediated Tat phosphorylation disrupts Tat-cyclin T1 interaction. (A) Fully phosphorylated or unphosphorylated Tat (His-Tat) was incubated with HeLa cell extracts at 4°C for 3 h, immunoprecipitated with anti-CycT1 antibody (Santa Cruz), and assessed by Western blotting (WB) and autoradiography (AR). (B) CycT1-mediated enhancement of Tat activity is abrogated by interferon treatment which blocks Tat-CycT1. pFlag-tat, pFlag-pkr, CycT1-expressing plasmid, and pLTR-luc reporter genes were cotransfected together with a pCMV-β-Gal transfection control into HeLa (eIF2α^{CA}) cells. After 48 h, luciferase activities were assessed in triplicates from each sample, normalized with the transfection control, and then represented as means ± SD (*n* = 3). *, *P* < 0.01; **, *P* < 0.05. (C) GST-tagged wt and mt Tat proteins were incubated with HeLa cell nuclear extracts and subjected to GST pulldown assay followed by Western blotting. Input GST-Tat proteins were stained (bottom). (D) Co-IP of CycT1 and wt and A/D-mt Tat *in vivo*. HEK293 cells were transfected with Flag-tagged wt or D/A-mt Tat plasmids (pCDNA3-Flag-tat). After 48 h, cell extracts were immunoprecipitated with anti-Flag antibody and analyzed by Western blotting assay. (E) Upper panels, pHA-PML-IV (red) and pEGFP-cyclin T1 (green) plasmids were cotransfected with or without the Flag-Tat (wt) plasmid into U2OS cells. Cells were stained with phycoerythrin (PE)-conjugated MAb. Lower panels, U2OS cells were transfected with Flag-tagged wt or mt Tat plasmid (pCDNA-Flag-tat) and pEGFP-cyclin T1, fixed, and stained with PE-conjugated anti-Flag MAb. Images were taken with a confocal microscope.

when Tat was preincubated with PKRa, it was unable to shift TAR RNA in an EMSA (Fig. 7C). In SDS-denaturing gel experiments, the non-TAR-bound free Tat in Fig. 7C was found to be strongly phosphorylated by PKRa, depending on the preincubation time (Fig. 7D). These data strongly suggested that PKR-mediated Tat phosphorylation impaired Tat binding to TAR RNA, probably

due to a conformational change of TAR-binding sites on Tat. These findings were further supported by additional EMSAs with TAR RNA and Tats with D substitutions at PKR target Ser/Thr sites. Tat-TAR interactions were significantly attenuated by accumulation of D mutations on Tat and were almost abolished by combined 23-40-46-62-68 D mutations (Fig. 7E).



PKR-mediated Tat phosphorylations inhibit Tat-cyclin T1 (P-TEFb) interactions. The interaction between cyclin T1 (CycT1) and HIV-1 Tat is well established with regard to efficient transcriptional elongation of viral RNAs (42, 43). To determine the effects of Tat phosphorylation on the Tat-CycT1 interaction, we performed co-IP assays. Control recombinant Tat was coimmunoprecipitated with endogenous CycT1, while Tat which had been fully phosphorylated by PKR was not (Fig. 8A). Tat-CycT1-mediated reporter (LTR-luc) expression was significantly downregulated by coexpression of PKR (Fig. 8B). These data suggest that PKR-mediated Tat phosphorylation disrupts Tat-CycT1 interactions. In a GST pulldown assay, T23D- and T40D-mt Tats were unable to pull down endogenous CycT1, while corresponding A mutants were still effective in the pulldown of CycT1 in HeLa cell extracts (Fig. 8C). In *in vivo* co-IP assays, 23D- and 40D-mt Tats did not immunoprecipitate CycT1, while 23A-, 40A-, and 46D/A mutants were as effective as wt Tat in co-IP with CycT1 (Fig. 8D). These results suggest that T23 and/or T40 is involved in the interaction with CycT1 and that PKR-mediated Tat phosphorylation at one or both of these sites disrupted Tat-CycT1 interactions.

CycT1 has been reported to physically interact with promyelocytic leukemia (PML) nuclear bodies, and Tat expression redistributes CycT1 out of PML bodies via the Tat-CycT1 interaction (44). Consistent with previous reports, EGFP-CycT1 fluorescence was diffused in the nucleoplasm in the presence of wt Tat (Fig. 8E, upper panels). Similar CycT1 diffusion was observed in cells expressing 23A-mt, 40A-mt, 46A-mt, or 46D-mt Tat but not in cells expressing 23D- or 40D-Tat mutants (Fig. 8E, lower panels). Endogenous CycT1 speckles were also redistributed by wt Tat but were not by 23D- or 40D-Tat mutants (data not shown). These data strongly support our assertion that T23 and/or T40 phosphorylation impedes Tat-CycT1 interaction.

PKR plays a crucial role in the p53-mediated HIV-1 restriction via Tat phosphorylation. To examine the effects of Tat phosphorylation on HIV-1 replication, we constructed recombinant HIV-1 cDNAs harboring mt Tat by subcloning of cDNA fragment at unique NheI/NcoI sites after PCR-based mutagenesis and generated recombinant viruses by transfection of these cDNAs. In a reporter assay, luciferase activity was significantly reduced or completely abolished when HeLa(LTR-luc) cells were transfected with HIV-1 cDNAs encoding single D-mt or multiple D-mt Tat, respectively, while the luciferase activities of the single A/N-mt Tat-encoding HIV-1 cDNAs (except 23A-Tat) were similar to that of wt-Tat HIV-1 cDNA (Fig. 9A). The natural amino acid substitution of Asp (N) for Thr23 (T23N) on Tat, which is prevalent in African strains, has been reported to increase Tat activity (16). Among the recombinant HIV-1 cDNAs, only those with T23N *tat*

or T40A *tat* genes produced high titers of progeny viruses after transfection of cDNAs, while the recombinant cDNAs with single or multiple corresponding Tat D mutations produced significantly reduced or undetectable levels of progeny viruses in the same transfection experiments (Fig. 9B). We also performed a complementation assay by the cotransfection of Tat-defective HIV-1 cDNA and mt/wt-Tat-expressing plasmids. Single or multiple A-mt Tats at PKR target sites were much more effective than compatible D-mt Tats for the production of Tat-defective HIV-1 (Fig. 9C). The replication capacity of wt HIV-1 was significantly inhibited by ectopic overexpression of PKR, while that of recombinant HIV-1 progeny viruses possessing Tat with 23N, 40A, or 23N40A substitutions was not as significantly inhibited compared to that of wt HIV-1 under the same conditions, even in the eIF2 α^{CA} cells (Fig. 9D). In contrast, HIV-1 (HXB2) replication was markedly enhanced by PKR knockdown and/or p53 knockdown (Fig. 9E). However, the HIV-1 replication in PKR^{KD} cells was not additionally increased by p53/PKR double knockdown (Fig. 9E), suggesting that p53-mediated host restriction of HIV-1 replication is mostly dependent on PKR activity in infected cells. To observe the physiological effects of p53 and PKR on the active replication of HIV-1 in infected T cells, syncytium formation was measured in HIV-1_{IIIB}-infected p53^{KD} or PKR^{KD} C8166 cells. The number of syncytia was also substantially enhanced in PKR^{KD} (sh-PKR) or p53^{KD} (si-p53) cells compared to that in normal (si-con) cells (Fig. 9F). In good agreement with the results shown in Fig. 9F, treatment of HIV-1-infected cells with 2-AP enhanced HIV-1 (HXB2) replication in p24 ELISA (Fig. 9G). All our experimental results strongly suggest that cellular PKR plays a crucial role in the p53-mediated host restriction of HIV-1 replication via Tat phosphorylation and inactivation in three different ways, as summarized in Fig. 10.

DISCUSSION

HIV-1 infections are detrimental to host survival and reproduction, and hosts have evolved numerous restriction mechanisms to protect themselves from HIV-1 replication (3, 45). It has been evidenced that p53 may play an important role in HIV-1 restriction. p53 was first suggested to inhibit HIV-1 replication by direct binding to the TFIID and inhibits HIV-1 LTR promoter activity (7). One year later, it was reported that p53 suppresses Tat-mediated LTR transcription (6). Recently, it was found that overexpression of wild-type p53 prevents the phosphorylation of serine 2 in the carboxyl-terminal domain (CTD) of RNA polymerase II on the LTR (8). It has also been reported that HIV-1 infection upregulates p53 expression and activation in primary CD4⁺ T cells (9, 10) and activates DNA-dependent protein kinase, leading to CD4⁺ T cell depletion (46). The combined reports indicate that

5 μ g of HXB2rTat cDNA (Tat-defective HIV-1) by electroporation. Five days after electroporation, HIV-1rTat titers in the supernatants were assessed by p24 ELISA (upper panel) and intracellular HIV-1 was assessed by Western blotting (lower panels). (D) C8166 cells were cotransfected with pcDNA3-Flag-PKR and pSLX-Myc-eIF2 α 51A plasmids. After 24 h, cells were infected with wt- or mt-Tat-expressing recombinant HIV-1 for 48 h. Cells were harvested and subjected to Western blot analysis. (E) p53^{KD} (si-p53) and/or PKR^{KD} (sh-PKR) C8166 cells were infected with HIV-1 at an MOI of 1 for 48 h. HIV-1 titers in the infected cells (upper panels) and the culture supernatants (lower panels) were assessed by Western blotting and p24 ELISA (means \pm SD, $n = 3$). **, $P < 0.01$ (control versus knockdown cells). (F) PKR^{KD} or p53^{KD} C8166 cells were infected with HIV-1_{IIIB} at an MOI of 1. Upper panels, the numbers of syncytia counted in 3 different cultures at 48 h postinfection are represented as means \pm SD (upper). *, $P < 0.05$; **, $P < 0.01$. Lower panels, cells were harvested and subjected to Western blot analysis with anti-p24 antibody. (G) C8166 cells were infected with HIV-1 (HXB2) at an MOI of 1 for 1 h, and then cultured in the presence or absence of 2 mM 2-AP. The HIV-1 titer in culture supernatants was assessed using a p24 ELISA kit (Perkin-Elmer) and represented as means \pm SD ($n = 3$) (upper panel), and HIV-1 p24 in cell lysates was assessed by Western blotting with anti-p24 antibody (lower panel). *, $P < 0.05$; **, $P < 0.01$ (2-AP-treated versus untreated [UT] cells).

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interaction, as shown in Fig. 8C to E, resulting in the inhibition of HIV-1 replication (Fig. 9B). Our findings are, to a certain extent, consistent with previous reports showing that the C22G and K41A substitutions on Tat disrupt Tat-CycT1 interaction *in vitro* (49–51). The phenotypes of PKR-mediated Tat phosphorylation and inactivation in transfected cell lines were quite dramatic. However, viral replication was not affected very much as shown in the Tat functional study, likely because of other factors in the infected cells.

Contrary to our expectations, T23A substitution, to avoid PKR-mediated Tat phosphorylation at T23, did not improve Tat activity but rather impaired it (Fig. 5A and 9A), with a reduction in HIV-1 replication (Fig. 9B and C). This is presumably due to the suboptimal conformational changes of Tat by T23A substitution, which may damage normal Tat activity. Instead, the recombinant HIV-1 cDNAs possessing a *tat* gene encoding the natural T23N substitution, which is dominant among African strains (Fig. 5E), produced a high titer of recombinant progeny viruses in the transfection experiments (Fig. 9B). The HIV-1 cDNA with 23N40A *tat* did not produce as much progeny virus as control cDNA (Fig. 9B, lower panels), suggesting that double mutation would be detrimental rather than synergistic, even though each single mutation (T23N and T40A) improved Tat activity. The HIV-1 cDNAs possessing a *tat* gene encoding the S46A substitution produced a significantly reduced level of progeny viruses (Fig. 9B), even though S46A Tat showed strong Tat activity in a reporter assay (Fig. 9A). This might be due to the disturbance of the Kozak sequence of the overlapping gene *rev* during S46A Tat mutagenesis, as S46 on Tat is located right above the *rev* initiation AUG codon. Several reports have shown that the strength of upstream AUGs can influence initiation at downstream AUGs in HIV-1 transcription (52–54). A subset of *env* mRNAs carry the upstream *rev* and *vpu* initiation codons, and mutation of a weak upstream HIV-1 *rev* Kozak sequence to a strong initiating sequence resulted in decreased leaky scanning and poor initiation at the downstream *vpu* AUG (55).

It has been previously reported that Tat proteins from African strains are much more active than those from Europe and the United States (15), and the natural amino acid substitution T23N on Tat is present in a broad spectrum of African strains that are fast replicating (16). These reports support our findings that PKR targets five Ser/Thr sites on Tat that are highly conserved in HIV-1 subtype B strains (Fig. 4D), such as Bru (56) and Jr (57), which are prevalent in the United States and Europe. In contrast, strains Eli and Mal from Zaire (58) and other clade A, C, D, and G strains demonstrated strong polymorphisms at the corresponding sites, with the exception of S46 (Fig. 5D and E). These data suggest that the replication capacity of HIV-1 strains is, at least in part, associated with the susceptibility to p53/PKR-mediated Tat phosphorylation at five specific Ser/Thr sites. Tat phosphorylation by PKR inhibits Tat activity, leading to restriction of HIV-1 replication after infection. Therefore, amino acid substitutions at PKR target sites on Tat would be an attractive strategy for HIV-1 to escape p53/PKR-mediated host restriction in African strains.

HIV-1 Tat is a potent *trans*-activator essential for HIV-1 replication. It has been suggested that p53 suppresses Tat functions. Here we report for the first time, to our knowledge, the detailed molecular mechanisms underlying p53-mediated Tat inactivation, followed by inhibition of HIV-1 replication. We found that p53-mediated Tat inactivation could be attributed to PKR-mediated

Tat phosphorylation at five specific Ser/Thr sites adjacent to the ARM, which decreased nuclear localization of Tat, Tat's ability to bind TAR RNA and P-TEFb, and Tat's transactivation capacity. Our findings may contribute to further research focusing on the investigation of potential therapeutic targets for HIV-1.

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We declare that we have no conflict of interest.

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